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Structural Characterization of the Interdomain Features of the Estrogen Receptor

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INTRODUCTION:

The nuclear receptor (NR) superfamily in humans contains 48 distinct ligand-activated transcription factors in humans. These receptors rely on inter-connected functional domains to mediate their function in transcriptional regulation. The N-terminal A/B domain of the NR family is poorly conserved in size and sequence. The DNA-binding domain (DBD) is highly conserved resides in the center of the polypeptide and binds to DNA response elements upstream of target genes. A hinge region connects the DBD to the ligand binding domain (LBD). This region is not conserved in size or sequence. The LBD is responsible for binding to the hydrophobic ligands, consisting of steroids or dietary lipids, as well as to distinct coregulator proteins. The isolated DBDs and LBDs of the NR family have been studied by X-ray crystallography in many cases. However, the only existing 3-D structure of any intact nuclear receptor is that of the PPAR/RXR complex, which we published last year. In the case of the Estrogen Receptor (ER)-alpha, crystals structures are available for the LBD and DBD as single domains, and again unavailable with the intact protein. Therefore, there is no conceptual framework to help understand their domain-domain interactions and how these interactions shape the overall physiology and pharmacology of the Estrogen Receptor, including its response to drugs used for breast cancer, such as Tamoxifen . We are employing the first studies to crystallize full-length nuclear receptors including the ER-alpha , ideally in several different states with ER bound ligands as well as in a DNA complex with the estrogen response element.

BODY:

Our efforts have focused on the expression and purification of high quality human ER-alpha protein for use in crystallization, which still remains the most challenging aspect of the project in many ways. To enhance the likelihood of obtaining high quality crystalline samples of a full-length ER protein for structure determination, we have recently added the production of the ER-beta protein to our efforts. The ER-alpha and ER-beta proteins are highly related in amin-acid sequence within their DBDs, hinge and LBDs, but vary in sequence and size in their A/B regions (the ER-beta contains a shorter A/B region). Both receptors contribute to the physiology of estrogen action and are important drug targets to consider for breast cancer therapy. We have expressed and purified the full-length hER proteins this past year, concentrating on an *E. coli* expression system instead of the SF-9 system previously employed.

The product that comes out of the purification process from both ER expression systems appears to correspond to the expected molecular size (see Figure 1 and 2). More thorough analysis on the ER-alpha purified protein has shown that the N-terminal sequence is intact as well. Both proteins are made with a hexa-histidine tag at their N-termini, and purified through three separate chromatographic steps initially (his-bind resin, Q-sepharose, S-sepharaose) and then complexed with duplex ER response element DNA (ERE, see Figure 3), and further purified as the DNA bound complex on a gel-filtration column. The ligand for ER is present throughout each of the purification steps in each buffer solution to maximize protein solubility and folding.

High affinity ligands such as tamoxifen (the drug used in breast cancer therapy), estradiol (The endogenous receptor ligand), and genestein (a soy flavinoid) are being used in our protein purification processes to stabilize the protein and allow successful purification, since the absence of ligand causes the protein to precipitate in the purification. In the presence of ligands added to large bacterial cultures (multi-liter), as well as in the purification steps, we can obtain enough protein for both crystallization trials and for Hydrogen-Deuteration exchange mass spectrometry which further assists us with obtaining the desired crystalline samples.

In terms of how we have been developing the usage of duplex DNA, we have now produced six distincly sized, highly purified ERE duplexes using synthetically made individual single strands. Each strand, following large scale synthesis, undergoes purification on high performance liquid chromatography using organic solvents, initially as a tritylated product, and subsequentely as a de-tritylated produce. All of our purified DNA duplexes consist of the same central bipartite ER response element (with palindromic copies of AGGTCA, separated by the three required base-pairs). These duplexes only vary in terms of their overall length and the type and size of their 5' and 3' base overhangs. Figure 3 shows the sequences and variations in the end products of this work.

Our efforts this past year have been to produce very high quality samples that we have now of full-length ER complexes bound in a 2:1 stoichiometry on ERE DNA. The protein qualities are shown in Figures 1 and 2, and DNA oligos shown in Figure 3. The complexes made are now available for detailed characterization by Hydrogen-Deuterium exchange mass-spectrometry. This technique will show us which regions of the ER proteins are undergoing high-rates of solvent exchange at the amino-backbone positions. Similar data, which we acquired previously on PPAR/RXR full-length complexes, helped us to redesign more optimum expression constructs for crystallization, since highly exchanging (correlating to disordered) regions of the polypeptides could be removed in the cloning process to generate more compact and ordered

proteins which give rise to a higher likelihood of crystallization and high angle diffraction. Without the information from H/D exchange mass spectrometry, there is no rational way to predict where to choose the N-terminal A/B region of the construct, and how much of it is best avoided due to poor folding and dynamic behavior. Moreover, the H/D exchange studies will greatly assist us in choosing which ER ligands and ERE DNA elements are most stabilizing in terms of producing the complex that will drive our crystallization efforts.

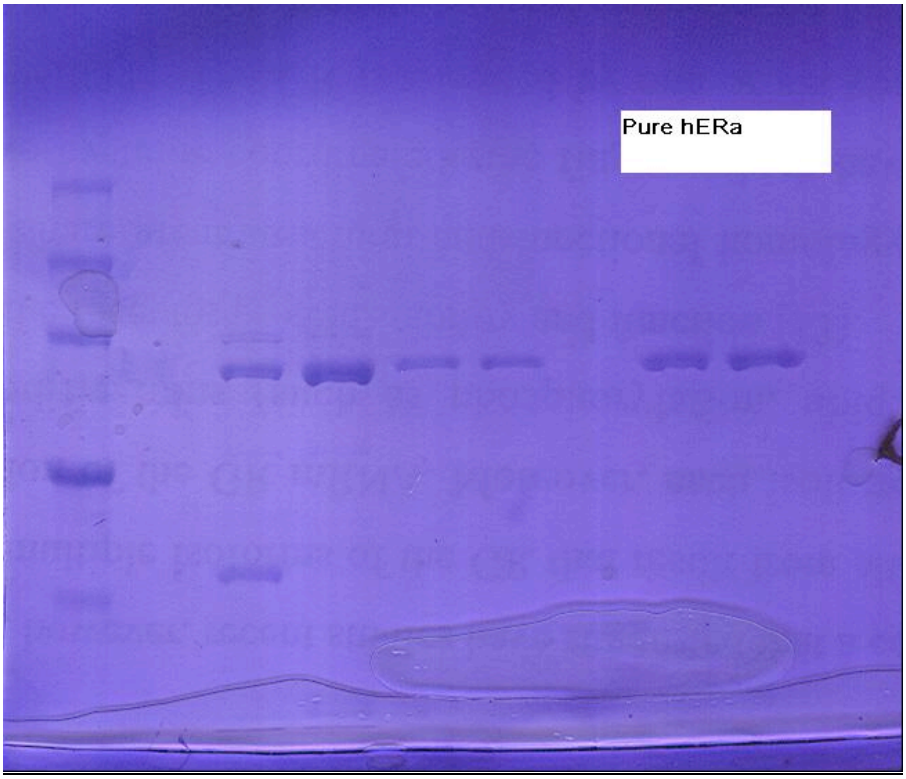

Figure 1: SDS-PAGE chromatography gel showing the fractions from the S-sepharose column eluting pure human ER-alpha protein (right side of gel), with molecular mark markers in the left-most lane. The full-length ER-alpha protein corresponds to the expected molecular mass based on sequence, and was expressed in *E. coli.*

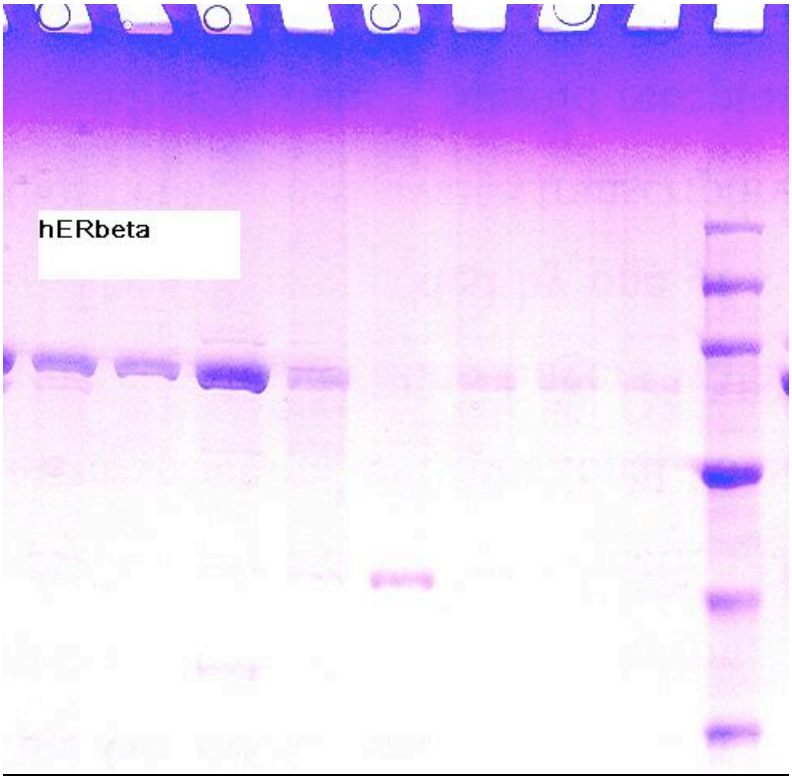

Figure 2: SDS-PAGE chromatography gel showing the fractions from the S-sepharose column eluting pure human ER-protein protein (left-most four lanes), with molecular mark markers in the right-most lane. The full-length ER-beta protein corresponds to the expected molecular mass based on sequence, and was expressed in *E. coli.*

5′-CC**AGGTCA**CAG**TGACCT**G-3′
3′ G**TCCAGT**GTC**ACTGGA**CC-5′ seq 1

5′-CTC**AGGTCA**CAG**TGACCTG**GA-3′ seq 2
3′ AG**TCCAGT**GTC**ACTGGAC**CTC-5′

5′-TGTC**AGGTCA**CAG**TGACCT**GAC-3′ seq 3
3′ CAG**TCCAGT**GTC**ACTGGA**CTGT-5′

5′-CGTC**AGGTCA**CAG**TGACCT**GAC-3′ seq 4
3′ CAG**TCCAGT**GTC**ACTGGA**CTGC-5′

5′-TGATC**AGGTCA**CAG**TGACCT**GATC-3′
3′ CTAG**TCCAGT**GTC**ACTGGA**CTAGT-5′ seq 5

5′-CGATC**AGGTCA**CAG**TGACCT**GATC-3′ Seq 6
3′ CTAG**TCCAGT**GTC**ACTGGA**CTAGC-5′

Figure 3: Sequences of DNA oligos corresponding to idealized estrogen response elements (EREs), but with different overall lengths and ending (overhang) sequences. Each of the duplexes shown represents molecules which we prepared in large quantities from synthesis, through four chromatography strands per single strand, and subsequently annealed to form the duplex. The darkened sequences correspond to the two half-sites, which would allow ER-alpha or ER-beta to bind as a homodimer as expected. The differences in overall length and overhang disposition are used as a variable in obtaining high quality crystals of the complex.

A previous strategy, based on selective removal of the portions undergoing rapid H-D exchange, had helped us get the PPAR-RXR/DNA crystals rather quickly. Furthermore, the eventual crystal structure of the PPAR-RXR/DNA complex we provided (published in *Nature* 2008) showed the details of domain-domain interactions that will be compared later to the features of the intact ER polypeptide, including a previously unknown interface between the DBD of one subunit and the LBD of the second (homo- or heterodimeric) polypeptide.

ACCOMPLISHMENTS:

a) Production of multiple intact ER-alpha protein complexes with duplex DNA – with different ligands on ER-alpha
b) Production of multiple intact ER-beta protein complexes with duplex DNA – with different ligands on ER-beta
c) Initiation of Hydrogen-Deuterium exchange mass spectrometry experiment on samples a) and b)

REPORTABLE OUTCOMES:

CONCLUSIONS:

The crystallization of the human Estrogen Receptor (alpha or beta) protein consisting of more than just one domain has been impossible to obtain over the past 15 years. This remains an important goal to pursue, as there is strong evidence suggesting that different domains in Ers are both physically and functionally connected and work in concert to mediate the responses to ligands, including drugs used in breast cancer therapy. Moreover, the structure determination of ER proteins should provide an important platform for design and understanding of future therapeutic drugs that can be used to treat breast cancer. Using similar strategies that have recently allowed us to successfully solved the first crystal structure of intact nuclear receptors PPAR-gamma and RXR-alpha as a heterodimer on DNA, we are working toward crystallization of a multi-domain version of either ER-alpha or ER-beta protein as a DNA complex. These strategies involve a) production of high quality homogenous ER proteins, b) production of various DNA duplexes with differences in overall length and overhang sequence that coulud favor growth of crystalline lattices, c) purification in the last step of the intact protein-DNA-ligand complex as a single entity, d) the reliance on two different forms of intact ER protein (alpha and beta) as crystallization variables, and e) the usage of hydrogen-deuterium exchange mass spectrometry to pinpoint regions of maximal disorder in the ER proteins and subsequently remove them from the protein expression constructs for more optimally diffracting crystals.

MANUSCRIPTS/ABSTRACTS.

Chandra, V., Huang, P., Hamuro, Y., Raghuram, S., Wang, Y. Burris, TP., Rastinejad F. Structure of the intact PPAR-gamma-RXR-alpha nuclear receptor compelx on DNA. *Nature* (2008) 456: 350-6.